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(54) Title: PROTEINACEOUS ADJUVANTS

(57) Abstract

A modulated immune response to an antigen is achieved by coadministering the antigen and a genetically-detoxified pertussis holotoxin, particularly one retaining its immunogenicity, to a host. The modulated immune response enables immunogenic compositions, including multivalent pediatric vaccines such as DTP, to be provided which produce a modulated immune response in the absence of extrinsic adjuvants such as alum. The adjuvanting effect achieved by the genetically-detoxified pertussis holotoxin enables at least the same level of adjuvanting effect to be achieved as previously attained by alum, without the undesirable side effects thereof.

PROTEINACEOUS ADJUVANTS

FIELD OF THE INVENTION

5   The present invention relates to the field of
immunology and is particularly concerned with
proteinaceous adjuvants., i.e. materials which modulate
immune responses to an antigen.

REFERENCE TO RELATED APPLICATION

10   This application is a continuation-in-part of
copending United States Patent Application Serial No.
08/258,228 filed June 10, 1994.

BACKGROUND OF THE INVENTION

Vaccines have been used for many years to protect
15 humans and animals against a wide variety of infectious
diseases.   Such conventional vaccines consist of
attenuated pathogens (for example, polio virus), killed
pathogens   (for example,   Bordetella   pertussis)   or
immunogenic components of the pathogen (for example,
20 diphtheria toxoid). Some antigens are highly immunogenic
and are capable alone of eliciting immune responses.
Other antigens, however, fail to induce, for example, a
protective immune response or induce only a weak immune
response.

25   Immunogenicity can be significantly improved if the
antigens are co-administered with adjuvants. Adjuvants
enhance the immunogenicity of an antigen but are not
necessarily immunogenic themselves.

Immunostimulatory agents or extrinsic adjuvants have
30 been used for many years to improve the host immune
responses to immunogenic compositions including vaccines.
Extrinsic adjuvants are immunomodulators which are
typically non-covalently linked to antigens and are
formulated to enhance the host immune responses. Thus,
35 adjuvants have been identified that enhance the immune
response to antigens delivered parenterally. Some of
these adjuvants are toxic, however, and can cause

undesirable side-effects, making them unsuitable for use
in humans and many animals.   Indeed, only aluminum
hydroxide and aluminum phosphate (collectively commonly
referred to as alum) are routinely used as adjuvants in
5   human and veterinary vaccines.   The efficacy of alum in
increasing antibody responses to diphtheria and tetanus
toxoids is well established and, more recently, a HBsAg
vaccine has been adjuvanted with alum.   While the
usefulness   of   alum   is   well   established   for   some
10   applications,   it has limitations.   For example, it is
ineffective for influenza vaccination and inconsistently
elicits a cell mediated immune response.   The antibodies
elicited by alum-adjuvanted antigens are mainly of the
IgG1 isotype in the mouse, which may not be optimal for
15   protection by some vaccinal agents.

Furthermore, studies in rats have demonstrated that
alum acts as an IgE adjuvant (ref. 1 – Throughout this
application,   various   references   are   referred   to   in
parenthesis to more fully describe the state of the art
20   to which the invention pertains.   Full bibliographic
information for each citation is found at the end of the
specification, immediately preceding the Claims.   The
disclosure   of   other   references   are   incorporated   by
reference into the present disclosures.   Studies with
25   tetanus and diphtheria toxoid vaccines also indicate that
alum adsorption of vaccines induces IgE antibodies in
humans (refs. 2, 3, 4).   Therefore, although the
inclusion of an aluminum salt in a vaccine formulation
may improve its immunogenicity and potency, the fact that
30   it can induce local granulomas and IgE antibodies which
may   contribute   to   hypersensitivity   reactions   warrants
careful examination of the practice of alum-adsorption of
vaccines for human and animal use.

Some characteristics of desirable adjuvants include:
35   (1)   a lack of toxicity;

(2) an ability to stimulate a long-lasting immune response;

(3) simplicity of manufacture and stability in long-term storage;

(4) an ability to elicit both cellular and humoral immune responses to antigens administered by various routes, if required;

(5) synergy with other adjuvants;

(6) a capability of selectively interacting with populations of antigen presenting cells (APC);

(7) an ability to elicit appropriate T_H1 or T_H2 cell-specific immune responses;

(8) an ability to selectively increase appropriate antibody isotype levels (for example, IgA) against antigens; and

(9) that they do not contribute to hypersensitivity reactions.

Of relevance to the present invention is a discussion of the development of pertussis vaccines presented below.

Thus, pertussis or whooping cough is a serious respiratory disease caused by the infection of the respiratory tract by the gram negative organism <u>Bordetella pertussis</u>. Pertussis is a major cause of childhood morbidity and is implicated in 360,000 deaths annually (ref. 5). The most effective method of control of the spread of the disease has proven to be the use of widespread immunization programs. The whole cell pertussis vaccine which was shown to have clinical efficacy in the 1950's, has been effective in controlling pertussis epidemics (refs. 6, 7, 8). The value of the vaccine was illustrated when Japan, Sweden and Great Britain abandoned routine childhood pertussis immunization. Shortly thereafter, these countries experienced major epidemics of pertussis (refs. 9, 10, 11, 12).

Although the whole cell pertussis vaccine is effective in preventing the incidence and spread of disease, the acceptance and uptake of the vaccine has been limited due to reports of vaccine associated adverse
5   effects. Therefore, an impetus for the creation of a non-reactogenic, effective and well defined acellular component pertussis vaccine was created. One of the key features of the acellular vaccine is the chemically detoxified pertussis toxin (PT) component. The presence
10 of native pertussis toxin in the whole cell vaccine has been a source of concern as studies in animal models have shown that it can induce lymphocytosis, histamine sensitization, potentiation of anaphylaxis and IgE antibodies, enhancement of insulin secretion and many
15 other systemic effects (ref. 13). The acellular pertussis vaccines differ with respect to the combinations and quantities of _Bordetella pertussis_ antigens included in the vaccines but the key antigens include the agglutinogens, pertactin, filamentous
20 hemagglutinin (FHA) and pertussis toxin (PT). Although the acellular vaccine has been demonstrated to be immunogenic and of comparable efficacy to the whole cell vaccine, it has not been as effective in preventing bacterial colonization (ref. 14). In addition, the
25 results from a Swedish field trial comparing acellular and whole cell pertussis vaccines indicated that the formaldehyde inactivated pertussis toxin present in the acellular vaccines showed evidence of reversion to toxicity (ref. 15). Therefore, other methods of
30 inactivating the pertussis toxin molecule were required.

To overcome the drawbacks of chemical detoxification, several groups developed genetically detoxified pertussis mutant holotoxin molecules (refs. 16, 17, 18, 19, 20, 21). A promising candidate was the
35 K9G129 mutant. Not only was the immunogenicity of the molecule retained, but the toxicity of this recombinant

5

toxin was greatly diminished (refs. 18, 19, 21). In
addition, immunization with the K9G129 mutant stimulated
both humoral and cellular pertussis antigen specific
responses (ref. 22). Although many clinical trials base
5   the evaluation of the immunogenicity of a vaccine solely
on the antibody response following immunization, studies
indicate an important role for cellular immunity in
protection against this disease. In animal models, the
cellular immune response has been demonstrated to be
10   important in the protective response against pertussis as
the adoptive transfer of cells from convalescent animals
into sublethally irradiated animals conferred protection
from challenge with Bordetella pertussis organisms while
the passive transfer of immune serum did not (refs. 23,
15   24). A retrospective study in humans indicated that cell
mediated immunity to Bordetella pertussis correlated with
a positive history of pertussis (ref. 25). Following
natural pertussis infection in humans, both an antibody
and cellular immune response are observed (ref. 26).
20   However, immunization with either the whole cell or
acellular component vaccines resulted in variable
pertussis antigen-specific cellular immune responses
(refs. 27, 28). It appeared that the chemical
detoxification of the pertussis toxin component destroyed
25   its T cell immunogenicity while the antibody responses
were unaffected (ref. 26). Therefore, only the
genetically detoxified pertussis toxin molecule could be
used to stimulate both a cellular and humoral immune
response.
30   The use of the recombinant PT mutant, K9G129, as a
pertussis vaccine component has been well described. A
number of different forms of the vaccine have been
suggested. Two formulations have been evaluated in
humans. The first formulation consisted of 15 µg of the
35   PT mutant which was alum-adsorbed with a total of 0.5 mg
of alum per dose (refs. 22, 29) while the other

formulation contained 7.5 µg of the K9G129 mutant as well
as 10 µg FHA and 10 µg pertactin and was also alum
adsorbed (ref. 30). These studies indicated that the
genetically detoxified pertussis vaccine candidate was
5 not only safe, immunogenic and could induce a cell
mediated response, but, when combined with the FHA and
pertactin antigens, it also provided better protection in
the intracerebral challenge test than a chemically
detoxified component pertussis vaccine (ref. 30). Other
10 suggested formulations include a formaldehyde-treated
K9G129 component (ref. 31) and a cellular vaccine derived
from a strain of Bordetella pertussis producing the
genetically inactivated K9G129 pertussis toxin molecule
(ref. 32). The formaldehyde treatment of the K9G129
15 molecule altered the immunogenicity of the molecule as
lower amounts of specific antibodies were induced. The
protective ability of the molecule was also decreased as
it was less effective in the intracerebral challenge
assay (ref. 32). However, the recombinant cellular
20 vaccine derived from the K9G129 producing strain proved
to be as effective as the whole cell pertussis vaccine
(ref. 32).

Although the preceeding formulations demonstrate the
advantages of improved safety and efficacy associated
25 with the use of a genetically detoxified pertussis toxin
molecule, they do not address the adverse effects of DPT
(diphtheria, pertussis and tetanus) vaccination not
associated with the pertussis molecule component (refs.
33, 46). All of the stated formulations involved the use
30 of either 0.3 mg of aluminum phosphate (ref. 32) or 0.5
mg aluminum hydroxide (refs. 29, 30). Aluminum salts
were introduced into the DT and DPT vaccine formulations
as an adjuvant that would potentiate strong antibody
responses when the levels of the toxoids or the numbers
35 of Bordetella pertussis organisms were decreased to avoid
adverse reactions (refs. 34, 35) and alum is now

routinely used in these vaccines as an adjuvant.
However, years of field experience with these adsorbed
pertussis vaccines and studies (refs. 36, 37) have
demonstrated that, although they contained less of the
5   identified reactogenic vaccine components, local
reactions were nonetheless precipitated (refs. 38, 39,
40, 41, 42). Histopathological examination of local
abscesses produced following vaccination revealed
aluminum hydroxide inclusions in giant cells (ref. 38).
10   Investigation into the frequency of such granulomas
indicated that they were associated with the aluminum
content in the vaccine as placebo immunized groups which
received only the aluminum fraction of the vaccine,
exhibited abscess formation at a similar reaction rate
15   (ref. 43). Further evidence in support of the role of
aluminum in these local reactions was derived from
studies comparing aluminum adjuvant adsorbed and plain
cholera and tetanus vaccines (refs. 44, 45). Deep
innoculation of the vaccine into the muscle decreases the
20   incidence of these abscesses but although improved
techniques can prevent the formation of abscesses (ref.
39), the potentiation of IgE responses by aluminum salts
is not affected.

It would be advantageous to provide immunogenic
25   compositions having modulated immune responses to the
constituent antigens without the disadvantages of local
toxicity and contribution to hypersensitivity of prior
art extrinsic adjuvants.

SUMMARY OF INVENTION

30   The present invention relates to avoiding the
problems associated with the use of alum as an adjuvant
in immunogenic compositions by employing a genetically-
detoxified pertussis holotoxin, which itself may be
immunogenic, to effect modulation of an immune response
35   to a non-Bordetella antigen.

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While the elimination of alum from vaccine formulations could have been an approach to address to the problems associated therewith, as noted above, alum was included in vaccine formulations to provide an
5   enhanced immune response to the antigens in the formulation. Elimination of alum, therefore, would be expected to lead to a less effective formulation and would be unlikely to have been proposed.

However, the genetically-detoxified pertussis
10 holotoxin surprisingly provides a modulation of the immune response of a non-Bordetella antigen which enables vaccine formulations and other immunogenic compositions to be provided which exhibit immune responses at least equivalent to those achieved by adjuvanting with alum.

15 Accordingly, in one aspect of the present invention, there is provided an immunogenic composition, which comprises a genetically-detoxified pertussis holotoxin, and at least one other, non-Bordetella, antigen, wherein said genetically-detoxified pertussis holotoxin is
20 present in an amount sufficient to modulate an immune response to said other antigen in the absence of an extrinsic adjuvant.

The immune response which is modulated by the presence of the genetically-detoxified pertussis
25 holotoxin may be humoral and/or a cellular immune response. In particular, the modulated immune response may be an enhanced IgG and/or cellular response to the other antigen.

The at least one other, non-Bordetella antigen
30 present in the immunogenic composition may provide a protective immune response to at least one pathogen, which may be a bacterial, viral or parasitic pathogen. The antigen may be selected from a wide range of pathogens. Representative pathogens include
35 Corynebacterium diphtheriae, Clostridium tetani, paramyxoviridae, haemophilus, influenza, hepatitis,

meningococci, streptococci, schistosoma and trypanosome.
The antigen also may be selected from cancer-associated
antigens, particularly melanoma, bladder, lung, cervical
and prostate cancer antigens.

5   The at least one other non-Bordetella antigen may
comprise inactivated tumor cells or membrane fractions
thereof. Tumor cells may be removed from a cancerous
host and then inactivated in any convenient manner, for
example, by irradiation or chemical inactivation. The
10   inactivated cells and/or membrane fraction thereof then
are mixed with the genetically-detoxified holotoxin to
provide an immunogenic composition according to the
invention. Such composition then may be administered to
a naive (i.e. non-cancer burdened) host to confer
15   prophylactic protection against tumor development. In
addition, such composition may be administered to a
tumor-burdened host to promote an anti-tumor immune
response in the host.

       The genetically-detoxified pertussis holotoxin may
20   itself be immunoprotective but the immunomodulating
effect thereof may be obtained in the absence of an
immune response to the holotoxin. The provision of
genetically-detoxified pertussis holotoxins is described
in U.S. Patents Nos. 5,085,862 and 5,221,618, assigned to
25   the assignee hereof and the disclosures of which are
incorporated herein by reference.

       The term "genetically-detoxified" as used herein has
the same meaning as in the aforementioned U.S. Patents
Nos. 5,085,862 and 5,221,618, namely a pertussis
30   holotoxin mutant which exhibits a residual toxicity of
about 1% or less, preferably less than about 0.5% of that
of the native toxin. The residual toxicity is determined
by CHO cell clustering assay and ADP-ribosyl-transferase
activity.

35   Such genetically-detoxified pertussis holotoxin may
be formed by mutagenesis of a nucleotide sequence coding

for the holotoxin, as described in the above-mentioned patents, so that at least one amino acid is removed or replaced. Multiple amino acids also may be removed or replaced.

5   The at least one amino acid which is removed or replaced may be present in the S1 subunit, specifically ARG^9, ARG^{13}, TRP^{26}, ARG^{58} and GLU^{129}. Where multiple amino acids are removed or replaced, it is preferred to remove or replace $(S1)ARG^9GLU^{129}$. When such mutation is

10   effected, it is preferred to replace ARG^9 by CYS^9 and GLU^{129} by GLY^{129}. (This specific mutant is sometimes depicted herein as K9G129.)

      Below are Tables 1a and 2 containing details of several mutations of pertussis holotoxin which may be

15   used as the genetically-detoxified pertussis holotoxin in the immunogenic compositions provided herein. (The Tables appear at the end of the descriptive text). Table 1b contains details of the in vivo characterization of the mutations of Table 1a.

20   The immunogenic compositions of the invention may contain at least one additional Bordetella antigen, including agglutinogens, FHA and pertactin.

      The immunogenic compositions provided herein may be formulated in the substantial absence of an extrinsic

25   adjuvant as a vaccine for human or animal administration. Such vaccine composition may exhibit a decreased IgE response.

      In one embodiment of the invention, the immunogenic compositions of the invention may be formulated in the

30   substantial absence of alum as a multivalent vaccine comprising the genetically-detoxified pertussis holotoxin in an immunoprotective form and amount along with diphtheria toxoid and tetanus toxoid as the other antigens, thereby providing a DTP vaccine formulation

35   from which alum or other extrinsic adjuvant is absent. Such DTP vaccine formulations usually also contain other

Bordetella antigens, including agglutinogens, FHA and pertactin.

In another aspect, the present invention provides a method of obtaining a modulated immune response to an antigen in a host, including a human, which comprises administering at least one non-_Bordetella_ antigen to the host, and coadministering to the host a genetically-detoxified pertussis holotoxin in an amount sufficient to modulate an immune response to the other antigen in the absence of an extrinsic adjuvant.

As noted above, the immune response may be a humoral and/or a cellular immune response and the modulated immune response may be an enhanced IgG and/or cellular immune response. The administration of the pertussis holotoxin and other antigen may be effected by administering to the host a composition as described above and provided according to the invention.

In a particular embodiment of the present invention antigens and adjuvants are coadministered. In this application the term "coadministration" means simultaneous administrations or administrations within a short time period such as between several minutes or hours and up to 3 days. The coadministrations may be at the same or different sites and by the same or different routes.

BRIEF DESCRIPTION OF THE FIGURES

The present invention will be further understood from the following description with reference to the Figures in which:

Figure 1 shows the potentiation of murine serum IgE antibody production by immunogenic compositions of the present invention;

Figure 2 shows the production of IgG antibodies by immunogenic compositions of the present invention;

Figure 3 shows the production of IgG antibodies by multivalent vaccines of the present invention; and

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Figure 4 shows the induction of cellular immune responses by multivalent vaccines of the present invention;

Figure 5 shows the T_h1 and T_h2 immune response phenotypes, as determined by cytokine profile in mice immunized with ovalbumin adjuvanted with PPD and genetically detoxified pertussis toxin PT (K9G129);

Figure 6 shows the T_h1 and T_h2 immune response phenotypes as determined by ovalbumin specific IgG2a and IgGE immunoglobulin profiles in mice immunized with PPD and genetically detoxified pertussis toxin, PT (K9GK9);

Figure 7 shows the number of tumor-free mice in immunotherapy experiments conducted herein. Six groups of mice with five mice per group were immunized with cell culture medium as a control and 10^4 live melanoma cells. The graph shows the number of mice that had no tumor thirty days after the challenge;

Figure 8 shows the tumor volumes as at day 30 from two of the six groups of mice plotted versus the number of days after challenge with 10^5 live melanoma cells. The open boxes with the dashed lines represent the five mice that were immunized with 10^4 irradiated cells alone (group 2) and the closed circles connected by the solid lines represent the five mice immunized with irradiated cells plus 1 µg of K9G129 (group 3); and

Figure 9 shows the tumor volumes at day 22. Mice were immunized with cell culture medium as a control and 10^4 irradiated melanoma cells alone or together with CFA or increasing concentrations of K9G129. The tumor volumes of each of five mice in the six groups are shown 22 days after challenge with 10^5 live melanoma cells. for the last three groups (mice receiving K9G129), the numbers by the arrows show the number of mice that were free tumors.

GENERAL DESCRIPTION OF INVENTION

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Referring to Figure 1, there is illustrated the
potentiation of serum IgE levels in mice immunized with
a chemically inactivated acellular alum-adjuvanted DTP
vaccine and a non-alum adjuvanted rDTP acellular vaccine
5   comprising the genetically-detoxified K9G129 PT analog.
The results indicate that the serum IgE levels in mice
immunized with the rDTP acellular vaccine were
significantly decreased (p<0.05) relative to the DTP
acellular vaccine containing the chemically toxoided
10   pertussis toxin molecule.

Referring to Figures 2 and 3 and Table 3, there is
illustrated a comparison of antigen specific antibody
levels produced following immunization of mice with an
alum adjuvanted DTP whole cell vaccine, an alum-
15   adjuvanted DTP acellular vaccine preparation and a DTP
vaccine containing the genetically-detoxified PT analog
K9G129 not adjuvanted with alum. The results shown in
Table 3 indicate that the anti-PT IgG response and the
CHO neutralization titres produced by the alum-adjuvanted
20   DTP acellular vaccine and the non-alum-adjuvanted
recombinant DTP acellular vaccine are equivalent. Thus,
although the alum-free recombinant formulation
demonstrated decreased IgE potentiating activity, it
nonetheless retained its effectiveness as a pertussis
25   vaccine as indicated by these anti-pertussis toxin IgG
titres. Further evidence of the retention of PT-specific
immunogenicity was obtained from CHO cell neutralization
assays.   Significantly, higher levels of anti-
agglutinogen 2+3 and anti-69 kD (pertactin) IgG
30   antibodies were detected in the serum samples from mice
immunized with alum-free recombinant formulation
(p<0.05).   The anti-FHA toxoid IgG responses were
equivalent in sera obtained from mice immunized with
either of the vaccines.
35   Figure 3 shows the anti-tetanus toxoid and anti-
diphtheria toxoid IgG antibody levels in sera of mice

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immunized with either the whole cell pertussis vaccine,
the defined component acellular DTP vaccine containing
the glutaraldehyde-detoxified pertussis molecule or the
alum-free recombinant acellular DTP vaccine. The
5   diphtheria and tetanus toxoid components in the latter
vaccine were also devoid of alum. The results indicate
that the acellular formulations induce significantly
higher anti-tetanus toxoid and particularly anti-
diphtheria toxoid IgG antibodies as measured by this
10 assay.   Furthermore,   the   alum-free   recombinant
formulation induced significantly higher anti-tetanus and
diphtheria toxoid IgG responses relative to the acellular
component DTP vaccine.

       The ability of the DTP vaccine formulations to
15 induce antigen-specific cellular immune responses was
evaluated _in vitro_ and the results are shown in Figure 4.
Splenocytes derived from mice immunized with either the
whole cell, acellular or alum-free recombinant acellular
DTP vaccines were cultured in the presence of the
20 specific vaccine antigens. The whole cell DTP vaccine
induced a significant anti-diphtheria toxoid cellular
response although not to the same degree as that
generated by the acellular component DTP vaccine. The
acellular component vaccine induced a relatively poor
25 pertussis antigen specific proliferative response with
the exception of the anti-69 kD and anti-diphtheria
toxoid responses. Of significance, however, was the
markedly increased antigen-specific proliferative index
induced   by   the   alum-free   recombinant   acellular
30 formulation in response to all the antigens tested. The
recombinant formulation clearly induced the highest
levels of antigen-specific proliferative responses of any
of the vaccines tested.

       In accordance with an embodiment of the invention
35 there is provided (as an example of an immunogenic
composition comprising a genetically-detoxified pertussis

holotoxin and at least one other non-_Bordetella_ antigen
wherein said genetically-detoxified pertussis holotoxin
is present in an amount sufficient to modulated an immune
response to said other antigen in the absence of an
5   extrinsic adjuvant) an alum-free acellular DPT vaccine
containing the genetically-detoxified PT analog K9G129.
Thus, although the alum-free formulation does not contain
an extrinsic (e.g. a mineral) adjuvant it does contain an
adjuvant nonetheless as the K9G129 mutant acts not only
10 as an antigen but as an adjuvant (i.e. a proteinaceous
adjuvant) as well. This property is apparent in the
pertussis antigen specific responses measured by enzyme
immunoassay (Fig.2).   Significantly higher anti-
agglutinogen 2+3 and anti-69 kD (pertactin) IgG responses
15 were evident in the serum samples derived from mice
immunized with the alum-free recombinant acellular
pertussis vaccine formulation while the FHA toxoid
specific responses were equivalent. Therefore, the new
formulation induced antibody responses specific for
20 pertussis vaccine antigens at levels that were either
comparable or greater than the levels induced by the
alum-adsorbed acellular pertussis vaccine.

The general intrinsic adjuvant activity of the
K9G129 mutant for other vaccine antigens (such as those
25 antigens present in human vaccines, such as paediatric
combination vaccines) was also evaluated. The tetanus
and diphtheria toxoid specific IgG responses in serum
obtained from mice immunized with either the alum
adsorbed whole cell or acellular pertussis vaccines or
30 the alum free recombinant vaccine were compared (Fig. 3).
The tetanus and diphtheria specific IgG titres in the
serum of mice immunized with the whole cell vaccine were
significantly lower than those observed in either of the
acellular DTP immunized groups. Although the alum-
35 adsorbed DTP vaccine induced significantly higher toxoid
specific responses relative to the whole cell vaccine

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immunized group, of all the vaccine formulations tested,
the alum-free formulation induced the highest titres of
toxoid specific IgG. Therefore, the adjuvant activity of
the K9G129 mutant is not restricted to only _Bordetella_

5   antigens. The invention extends to a multivalent vaccine
containing protective antigens for a plurality of
pathogens.

Vaccine preparation and use

        As indicated above, the present invention in one
10 embodiment provides immunogenic compositions, suitable to
be used as, for example, vaccines. The immunogenic
composition elicits an immune response by the host to
which it is administered including the production of
antibodies by the host. The immunogenic compositions
15 include at least one non-_Bordetella_ antigen in one
embodiment. This antigen may be an inactivated pathogen
or an antigenic fraction of a pathogen. The pathogen may
be, for example, a virus, a bacterium or a parasite. The
pathogen may be inactivated by a chemical agent, such as
20 formaldehyde, glutaraldehyde, β-propiolactone,
ethyleneimine and derivatives, or other compounds. The
pathogen may also be inactivated by a physical agent,
such as UV radiation, gamma radiation, "heat shock" and
X-ray radiation. Representive pathogens from which the
25 antigen may be derived include _Corynebacterium_
diphtheriae, _Clostridium_ _tetani_, paramyxoviridae,
haemophilus, influenza, hepatitis, meningococci,
streptococci, schistosoma and trypanosome.

        An antigenic fraction of a pathogen can be produced
30 by means of chemical or physical decomposition methods,
followed, if desired, by separation of a fraction by
means of chromatography, centrifugation and similar
techniques. In general, low molecular components are
then obtained which, although purified, may have low
35 immunogenicity, alternative antigens include cancer-
specific antigens including melanoma, lung, cervical,

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prostate and bladder cancer antigens. Alternatively, antigens or haptens can be prepared by means of organic synthetic methods, or, in the case of, for example, polypeptides and proteins, by means of recombinant DNA methods.

The immunogenic compositions may be prepared as injectables, as liquid solutions or emulsions. The antigens and immunogenic compositions may be mixed with physiologically acceptable carriers which are compatible therewith. These may include water, saline, dextrose, glycerol, ethanol and combinations thereof. The vaccine may further contain auxiliary substances, such as wetting or emulsifying agents or pH buffering agents, to further enhance their effectiveness. Vaccines may be administered by injection subcutaneously or intramuscularly.

Alternatively, the immunogenic compositions provided by the present invention, may be delivered in a manner to evoke an immune response at mucosal surfaces. Thus, the immunogenic composition may be administered to mucosal surfaces by, for example, the nasal, anal, vaginal or oral (intragastric) routes. Alternatively, other modes of administration including suppositories may be desirable. For suppositories, binders and carriers may include, for example, polyalkylene glycols and triglycerides. Oral formulations may include normally employed incipients, such as pharmaceutical grades of saccharine, cellulose and magnesium carbonate.

These compositions may take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 1 to 95% of the immunogenic compositions of the present invention.

The immunogenic compositions are administered in a manner compatible with the dosage formulation, and in such amount as to be therapeutically effective, protective and immunogenic. The quantity to be

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administered depends on the subject to the immunized, including, for example, the capacity of the subject's immune system to synthesize antibodies, and if needed, to produce a cell-mediated immune response. Precise amounts

5   of antigen and immunogenic composition to be administered depend on the judgement of the practitioner. However, suitable dosage ranges are readily determinable by those skilled in the art and may be of the order of micrograms to   milligrams.   Suitable   regimes   for   initial

10   administration and booster doses are also variable, but may   include   an   initial   administration   followed   by subsequent administrations. The dosage of the vaccine may also depend on the route of administration and will vary according to the size of the host.

15   The concentration of antigens in an immunogenic composition according to the invention is in general 1 to 95%. A vaccine which contains antigenic material of only one pathogen is a monovalent vaccine. Vaccines which contain antigenic material of several pathogens are

20   combined vaccines and also belong to the present invention.   Such   combined   or   multivalent   vaccines contain, for example, material from various pathogens or from various strains of the same pathogen, or from combinations of various pathogens.

25   Immunoassays

        In one embodiment, the immunogenic composition of the present invention are useful for the generation antigen-specific antibodies that are themselves useful in the specific   identification   of   that   antigen   in   an

30   immunoassay.   Such immunoassays include enzyme-linked immunosorbent assays (ELISA), RIAs and other non-enzyme linked antibody binding assays or procedures known in the art.   In ELISA assays, the antigen-specific antibodies are immobilized onto a selected surface; for example, the

35   wells of a polystyrene microtiter plate. After washing to remove incompletely adsorbed antibodies, a nonspecific

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protein, such as a solution of bovine serum albumin (BSA) or casein, that is known to be antigenically neutral with regard to the test sample may be bound to the selected surface. This allows for blocking of nonspecific

5   adsorption sites on the immobilizing surface and thus reduces the background caused by nonspecific bindings of antigens onto the surface. The immobilizing surface is then contacted with a sample, such as clinical or biological materials, to be tested in a manner conducive

10   to immune complex (antigen/antibody) formation. This may include diluting the sample with diluents, such as BSA, bovine gamma globulin (BGG) and/or phosphate buffered saline (PBS)/Tween. The sample is then allowed to incubate for from about 2 to 4 hours, at temperatures

15   such as of the order of about 25° to 37°C. Following incubation, the sample-contacted surface is washed to remove non-immunocomplexed material. The washing procedure may include washing with a solution such as PBS/Tween, or a borate buffer.

20   Following formation of specific immunocomplexes between the antigen in the test sample and the bound antigen-specific antibodies, and subsequent washing, the occurrence, and even amount, of immunocomplex formation may be determined by subjecting the immunocomplex to a

25   second antibody having specificity for the antigen. To provide detecting means, the second antibody may have an associated activity, such as an enzymatic activity, that will generate, for example, a colour development upon incubating with an appropriate chromogenic substrate.

30   Quantification may then achieved by measuring the degree of colour generation using, for example, a visible spectra spectrophotometer.

                            EXAMPLES

         The above disclosure generally describes the present

35   invention. A more complete understanding can be obtained by reference to the following specific Examples. These

Examples are described solely for purposes of illustration and are not intended to limit the scope of the invention. Changes in form and substitution of equivalents are contemplated as circumstances may suggest

5   or render expedient. Although specific terms have been employed herein, such terms are intended in a descriptive sense and not for purposes of limitations.

Example 1

This Example describes the formulation of vaccines.

10   The DPT whole-cell vaccine and an experimental component acellular vaccine were produced by Connaught Laboratories Ltd. The component acellular pertussis vaccine was alum adsorbed (1.5mg/dose) and consisted of 10 μg protein nitrogen of glutaraldehyde toxoided

15   pertussis toxin , 5 μg protein nitrogen each of FHA and agglutinogens 2 and 3 and 3 μg of pertactin along with 5 Lf of tetanus toxoid and 25 Lf of diphtheria toxoid per dose. The recombinant component vaccine also contained 5 μg protein nitrogen each of FHA and agglutinogens 2 and

20   3 and 3 μg protein nitrogen of pertactin in addition to 5Lf of tetanus toxoid and 25 Lf of diphtheria toxoid per dose. However, it varied from the other acellular vaccine in that it contained 20 μg protein nitrogen of the recombinant PT mutant holotoxin, K9G129, and was not

25   alum adsorbed. The K9G129 pertussis toxin molecule as well as the purified FHA, agg 2+3 and pertactin components were obtained individually from Connaught Laboratories Ltd.

Example 2

30   This Example describes immunization of animals.

Female BALB/c mice weighing 15 to 18 grams were obtained from Charles River Canada (St. Constant, Quebec). The mice were housed in microisolators and used in accordance with the guidelines set by the Canadian

35   Council on Animal Care (CCAC). The animals were specific pathogen free and the housing rooms were monitored for

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Murine Hepatitis Virus outbreaks through the use of
sentinel mice. Water was provided _ad libitum_ and the
diet was ovalbumin-free. The mice were immunized on Day
0 with the vaccine formulations in groups of six. A
5   booster dose of vaccine was administered on Day 21. On
Day 28 the animals were bled via jugular vein laceration
and splenectomized. The serum samples were stored at
-20°C until assayed.

Example 3

10   This Example describes antigen specific
immunoassays.

The vaccine antigen specific IgG responses were
determined by indirect EIA. The antigens of interest
were pertussis toxin, pertactin, filamentous
15 hemagglutinin, agglutinogens, as well as diphtheria and
tetanus toxoids. High binding capacity microplates
(Nunc) were coated with 4 μg/mL of each of the above
antigens in a volume of 50 uls/well of 50 mM carbonate
buffer pH 9.6. After an overnight incubation, the plates
20 were washed and successively blocked with a 0.1% solution
of bovine serum albumin (Sigma) for one hour at room
temperature. The excess block was removed and the
microplates were washed. The murine serum samples were
then serially diluted in PBS-Tween 20 (0.05%) and plated
25 out at a volume of 100 uls. The samples were incubated
overnight at 4°C. The antigen specific fraction of IgG
antibodies was detected by a peroxidase conjugated sheep
anti-mouse IgG conjugate (Jackson Laboratories). The
plates were developed with the TMB substrate as above and
30 were read at dual wavelengths of 450nm and 540 nm in the
Multiskan MCC 340 MKII microplate reader. Reactive
titres were defined as the dilution at which the
absorbance of the test sample was equivalent to the mean
plus three standard deviations of the negative control
35 absorbance values. The geometric means and 95%

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confidence intervals were calculated and the groups were compared using the Student's t-test.

Example 4

This Example describes the determination of
5   ovalbumin specific IgG subclass profiles.

Example 5

This Example describes total IgE immunoassays.

The serum total IgE levels were assessed by indirect EIA. Nunc immunoplates (Gibco/BRL) were coated at room
10   temperature overnight with a sheep anti-mouse IgE polyclonal antiserum (Serotec) diluted in 50 mM carbonate buffer pH 9.6. The next day the plates were washed in PBS containing 0.05% Tween 20 (J.T. Baker) and then blocked with 0.1% casein amino acids (Difco) for one hour
15   at room temperture. After the excess blocking solution was washed off the plates, the murine serum samples were serially diluted three-fold in the assay diluent and plates out onto the microplate at 100 uls per well. The samples were incubated overnight at 4°C. To detect the
20   bound IgE antibodies, a biotinylated rat anti-mouse Ige monoclonal antibody (Serotec) was added to each well at a concentration of 2ug/mL and incubated for one hour at room temperature. After washing, peroxidase conjugated streptavidin (Dimension Laboratories) was added to each
25   well. The amount of IgE bound to the wells was assessed by adding the enzyme substrate, 10% tetramethylbenzidine (TMB) (ADI Diagnostics) in 0.005% hydrogen peroxide water (Fisher Scientific). The reaction was stopped after ten minutes with 1M sulfuric acid (Fisher Scientific). The
30   absorbance of the wells was measured at 450 nm with a background correction at 540 nm on a Multiskan MCC 340 MkII microplate reader (Flow Laboratories). The serum IgE levels were quantitated by calibrating the sample absorbances against a standard curve generated by a
35   serially diluted IgE murine myeloma protein run on each plate. The geometric means and 95% confidence intervals

were calculated for each treatment group and the groups
were compared using the Student's t-test and p<0.05.

Example 6

5   This Example describes the determination of
ovalbumin specific IgGF immunoglobulins.

        The levels of IL-4 and IFN-γ were determined in a
sandwich EIA. Briefly, 96 well Nunc Maxisorp microplates
(Gibco/BRL) were coated overnight at room temperature
10   with cytokine monospecific rat monoclonal antibodies.
These antibodies were obtained from Pharmingen and
derived from the following respective clones: IL-4,
clone 11B11; IL-5; IFN-γ, clone R4-6A2. The monoclonal
antibodies were diluted to a concentration of 2 μg/mL in
15   50 mM carbonate buffer pH 9.6. The following day, the
plates were washed in PBS-Tween 20 0.05% (PBS-T) and
nonspecific binding sites were blocked by the addition of
a 1% bovine serum albumin (Sigma) solution diluted in
PBS-T.   Following incubation for one hour at room
20   temperature, the excess block was washed from the plates
and undiluted culture supernatants were added to the
wells in duplicate.   The appropriate recombinant
standards for each cytokine (recombinant IL-4 obtained
from Pharmingen, recombinant IFN-γ purchased from
25   Genzyme) were diluted to the appropriate concentrations
(initial concentration of 100 ng/mL or 1000 ng/mL for IL-
10 EIA) and serially diluted three-fold in RPM 1640
(Sigma) containing 10% fetal bovine serum (FBS). The
standards were plated out at 100 μls/well and the
30   microplates were incubated overnight at 4°C. After a
vigorous wash in PBS-T, the bound cytokines were detected
using a biotinylated monoclonal antibody specific to each
cytokine and diluted to a concentration of 2 μg/mL in
PBS-T. The antibodies were obtained from Pharmingen and
35   were derived from the following clones: IL-4 clone BVD6-
24G2; IFN-γ, clone XMG1.2. After a one hour incubation

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step at room temperature, a peroxidase conjugated
streptavidin preparation (Vector Laboratories) diluted to
a concentration of 500 ng/mL was added. A final wash was
performed following a one hour incubation at room
5   temperature of the streptavidin preparation. The plates
were developed by the addition of the substrate, 10% TMB
in 0.05% hydrogen peroxide (Fisher Scientific) water.
The reactions were allowed to proceed until suitable
colour intensity was reached and were stopped by the
10   addition of 100 μls/well of a 1M solution of sulfuric acid
(Fisher Scientific). The absorbances of the reaction
wells were read at dual wavelengths (450 nm and 540 nm)
on a Multiskan MCC 340 MkII (Flow Laboratories)
microplate reader.
15   The cytokine concentrations in the supernatants were
quantitated by calibrating the sample absorbances against
the absorbances of the standards of known concentrations
using the logistic curvefit algorithm to fit the curve
with a minimum correlation coefficient of 99.9%. The
20   ELISA+ software package (Meddata) was used to quantitate
the amounts of cytokines present in the supernatants
based on the standard curves generated on each plate.

Ovalbumin-specific IgE titres in the sera of
25   immunized mice were determined by use of an indirect
antigen capture EIA. Briefly, Nunc Maxisorp microplates
(Gibco/BRL) were coated with a rabbit anti-ovalbumin IgG
fraction (Cappel Laboratories). The plates were
incubated overnight at room temperature. The next day,
30   after washing in PBS-T, the plates were blocked with a
solution of 0.1% skimmed milk powder diluted in PBS-T for
one hour at room temperature. Next, a solution of
ovalbumin diluted to 10 μg/mL in 50 mM carbonate buffer
pH 9.6 was added to each well in 100 μl volumes.
35   Following a one hour incubation at room temperature, the
ovalbumin solution was washed off the plates. The murine

serum samples were then serially diluted three-fold in
PBS-T at an initial dilution of 1:40 and a final dilution
of 1:87480. 100ml samples were added per well and
incubated overnight at 4°C. After washing the next day,
5   the ovalbumin specific IgE antibodies bound to the plates
were detected using a biotinylated rat anti-mouse IgE
monoclonal antibody (Clone LO-ME-2, Serotec) diluted to
2 μg/mL in PBS-T. Following a further one hour
incubation at room temperature, this antibody was washed
10   off and peroxidase conjugated streptavidin (Vector
Laboratories) was added to each well at a concentration
of 500 ng/mL. The amount of ovalbumin-specific IgE in
the murine serum samples was detected by adding the
peroxidase substrate, 10% tetramethylbenzidine (TMB) (ADI
15   Diagnostics) in 0.005% hydrogen peroxide. The color in
the wells was allowed to develop for fifteen minutes and
the reactions were stopped by the addition of 100 μls of
1M sulfuric acid (Fisher Scientific). The absorbance of
the wells was measured in a microplate reader (Multiskan
20   MCC 340 Mk11, Flow Laboratories) at 450 nm with a reading
at 540 nm for background correction. Reactive titres
were defined as the last dilution at which the absorbance
value of the test sample was equivalent to the mean of
the absorbance values derived from a negative serum
25   control plus three standard deviations (134,139). The
geometric means were calculated on log transformed data
and expressed with 95% confidence intervals.

Example 7
30   This Example describes the determination of murine
cytokine profiles.
        The ovalbumin-specific IgG, IgG1 and IgG2a titres in
murine serum samples were measured by indirect EIA. In
the IgG2a assay, Nunc Maxisorp 96-well microplates
35   (Gibco/BRL) were coated with a rabbit anti-ovalbumin
polyclonal antibody IgG fraction (Cappell Laboratores)

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diluted in 50 mM carbonate buffer pH 9.6 and incubated
overnight at room temperature. Ovalbumin-specific IgG
and IgG1 responses were measured on microplates coated
directly with ovalbumin (Sigma) diluted to a
5 concentration of 10 μg/mL in 50 mM carbonate buffer. The
following day, the microplates were washed in PBS-T and
blocked for one hour at room temperature with a solution
of 0.1% skimmed milk powder diluted in PBS-T. After a
further washing step, a 10 μg/mL solution of ovalbumin
10 (Sigma) diluted in 50 mM carbonate buffer pH 9.6 was
added to the IgG2a specific assay. This antigen coat
was incubated for one hour at room temperature and was
followed by a washing step.

The next step in the assay required the
15 addition of the murine serum samples. In the IgG2a
assay, the serum samples were serially diluted three-fold
beginning at an initial dilution of 1:40 and ending at a
dilution of 1:87480. The IgG and IgG1 assays were
carried out with serum samples diluted three-fold
20 starting at an initial dilution of 1:360 and ending at a
final dilution of 1:787320. The serum samples were
diluted in PBS-T and added to the wells of the
microplates in 100 μl volumes. The plates were incubated
overnight at 4°C. The next day, the plates were washed
25 and the ovalbumin-specific IgG subclasses of antibodies
were detected with biotinylated rat anti-mouse IgG
conjugates specific for each IgG antibody subclass (IgG2a
conjugate, derived from clone R19-15 and obtained from
Pharmingen, IgG1 conjugate, derived from clone LO-MGI-2
30 and obtained from Serotec) while the IgG responses were
detected with a 1:50,000 dilution of a peroxidase-
labelled sheep anti-mouse IgG (Fcγ specific, Jackson
Laboratories). The conjugated monoclonal antibodies were
diluted to a concentration of 2 μg/mL and incubated for
35 one hour at room temperature. After washing, peroxidase
conjugated streptavidin was added to each well of the

plates containing a biotinylated conjugate at a
concentration of 500 ng/mL. The plates were incubated
for one hour at room temperature. The bound antigen-
specific IgG and IgG subclass antibodies were detected by
5   the addition of the peroxidase substrate, 10% TMB (ADI
Diagnostics) diluted in 0.005% hydrogen-peroxide (Fisher
Scientific). The reactions were allowed to proceed for
a period of ten minutes at which point they were
terminated by the addition of 1M sulfuric acid (Fisher
10 Scientific) to each well. The microplates were read on
a microplate reader (Multiskan MCC 340 MkII, Flow
Laboratories) at dual wavelengths of 450nm and 540 nm.
Reactive titres were defined as the last dilution at
which the absorbance of the test sample was equivalent to
15 the mean plus three standard deviations of the negative
control absorbance values. The geometric means were
calculated on log transformed data and expressed with 95%
confidence intervals.

20   Example 8
         This Example describes antigen-specific cellular
immune responses.
         Murine splenocytes were obtained from the vaccine
immunized BALB/c mice on Day 28. The spleens were
25 dissociated into a single cell suspension and washed
three times in RPMI 1640 media (Sigma). A cell count was
performed using the trypan blue exclusion method and the
cells were adjusted to a concentration of 2×10^6 cells/mL.
The antigens (pertussis toxoid, pertactin, FHA,
30 agglutinogens, and non alum-adsorbed diphtheria and
tetanus toxoids were diluted to a concentration of 5
µg/mL in RPMI 1640 media containing 10% fetal bovine
serum. The antigens were then serially diluted two fold
to a concentration of 78 ng/mL. The cells were then
35 added to each well at a final concentration of 1×10^5
cells/well. The cultures were left to incubate at 37°C

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in a 5% CO_2 incubator for 72 hours. At the end of this
period, the cells were pulsed with 0.5 μCi/well of
tritiated thymidine (Amersham) diluted in sterile PBS
(Sigma). After a further 18 hour incubation, the cells

5   were harvested onto glass fibre filter paper using a 96
well harvester (Canberra Packard) and the radioactive
counts were read on a Matrix 96 beta counter (Canberra
Packard.   The results were expressed as stimulation
indices which were calculated by dividing the means of

10 the test counts by the means of the background counts on
the plate. Each sample was assayed in triplicate.

Example 9

        This Example describes the ability of antibodies to
neutralize pertussis toxin in the CHO cell neutralization

15 assay.

        The ability of the antibodies induced by the
pertussis vaccines to neutralize pertussis toxin was
assessed in the CHO cell assay as described by Granstrom
et al. (ref. 47). The last dilution of antibody at which

20 no significant morphological effects could be seen was
defined as the neutralizing titer.   The results were
expressed as reciprocal neutralizing titers.

Example 10:

        This example illustrates the use of genetically-
25 detoxified pertussis holotoxin to confer prophylactic
protection against tumor development.

        Thus, the B16 mouse melanoma model (Ref. 54) was
used to assess the effectiveness of K9G129 as an adjuvant
in cancer immunotherapy. When C57B1/6 mice were injected
30 subcutaneously with live syngeneic B16-F1 strain of B16
melanoma cells, tumors appeared after about ten days and
progressively grew in an exponential manner.   Tumor
appearance was directly proportional to the dose of cells
injected, eg. tumors formed earlier when mice were
35 injected with 10^6 cells than with 10^4 cells. Tumors could
be delayed by immunizing the mice with B16 melanoma cells

that had first been irradiated with 10,000 rads.   This
delay was also dose dependent.   Immunizing with 10^6
irradiated cells caused a greater delay in tumor
appearance than immunizing with 10^5 irradiated cells,
5   when the mice were subsequently challenged with 10^5 live
cells.   Immunizing with 10^4 irradiated cells caused no
significant delay in tumor growth.

The effectiveness of K9G129 as an adjuvant was
tested by measuring its ability to delay tumor growth
10   when combined with 10^4 irradiated cells in an
immunization experiment.   Six groups of mice with five
mice per group were immunized with:

1) cell culture medium (control)

2) 10^4 irradiated cells

15   3) 10^4 irradiated cells + 1 μg K9G129

4) 10^4 irradiated cells + 5 μg K9G129

5) 10^4 irradiated cells + 10 μg K9G129

6) 10^4 irradiated cells + CFA (complete Freunds adjuvant)

The mice were boosted in the same manner two weeks later
20   and then two weeks after the boost they were challenged
with 10^5 live B16 melanoma cells.   The appearance of
tumors was monitored and the size of growing tumors was
measured with calipers, noting both the length and width.
The volume of the tumors was calculated by applying these
25   measurements to the formula for an ellipsoid.

K9G129 was effective in a dose dependent manner in
delaying the onset of tumor growth.   Thirty days after
the challenge with 10^5 live melanoma cells, there were no
mice without tumors in the groups that received no
30   immunization (group 1), irradiated cells alone (group 2),
or irradiated cells with CFA (group 6).   There were also
no tumor-free mice in the group that had received
irradiated cells with 1 μg of K9G129 (group 3).   However
there were two and four mice respectively that had no
35   tumor from the groups that had received irradiated cells
with 5 μg and 10 μg of K9G129 (groups 4 and 5) (Fig.7).

30

These results show a large delay in tumor appearance
mediated by the two higher concentrations of K9G129.

Even the lowest concentration of K9G129 used caused
a delay in tumor appearance. Four of five mice in group
5   3 (irradiated cells + 1 μg K9G129) had tumors appear
after tumors had begun to grow in the mice that had been
immunized with irradiated cells alone (group 2) (Fig.8).
The effectiveness of K9G129 in delaying tumor growth is
further demonstrated by comparing the tumor volumes of
10 individual mice in the various groups, 22 days after the
challenge with live melanoma cells. Tumors are non-
existent or their sizes are generally lower in mice that
were immunized with irradiated cells and K9G129 than in
mice immunized with irradiated cells alone or in
15 conjunction with CFA (Fig.9).

These results indicate that K9G129 can act as an
adjuvant in cancer immunotherapy to increase the immune
response towards tumor cells.

Example 11

20   This Example describes the generation of a Th1
response to an immunogen adjuvanted with the S1(K9G129)
Pertussis Toxin analogue.

One, of the key factors involved in the potentiation
of different immunoglobulin subclasses, including IgE, is
25 the presence of soluble mediators known as cytokines.
The control of IgE production is regulated by a variety
of cytokines which not only possess direct effector
functions such as the induction of immunoglobulin isotype
switching, but also act to cross-regulate the production
30 of other cytokines. In the mouse, IL-4 acts not only to
induce IgE and IgG1 isotype switching, but also acts to
inhibit the secretion of IgM, IgG3, IgG2b and IgG2a (Ref.
48). On the other hand, IFN-γ acts to stimulate the
production of IgG2a and IgG3 while inhibiting IgG1, IgG2b
35 and IgE synthesis (Ref. 48).

31

In an effort to organize and rationalize the multiple and cross-regulatory effects of cytokines, a system to describe the various patterns of cytokine secretion has been described (Ref. 49). Mosmann and
5   Coffman (Ref. 49) defined two distinct subsets of murine CD4^{+} T cells based on their differential patterns of cytokine secretion. Using long term T cell clones, they were able to show that one group of cells, defined as Th2, secreted IL-4, IL-5 and IL-10 while another group of
10 cells, defined as Th1, secreted IL-2, IFN-γ and TNF-β. These two distinct cytokine profiles were also correlated with immunoglobulin production in that Th1 clones provided help for B lymphocytes to produce IgG2a while Th2 clones promoted the secretion of IgG1 and IgE by B
15 cells (48,50,51). Later work by Romagnani and coworkers demonstrated the existence of these T cell subsets in humans as well (Ref. 52).

Although the initial differentiation of Th1/Th2 cytokine profiles was defined on the basis of in vitro
20 cytokine patterns of individual T cell clones, the definitions have been extended to describe the cytokine phenotypes resulting from immunization or infection (Ref. 48,53). These phenotypes are not as starkly polar as those observed in the original clonal analysis and are
25 defined by a variety of different cytokines. Thus, a Th1 phenotypic response is characterized by a significant increase in Th1-type cytokines (higher ratios of IFN-γ:IL-4) relative to Th2 immune response phenotypes. This classification also extends to the antigen-
30 specific immunoglobin subclass profiles where Th1 phenotypes present as higher IgG2a:IgE ratios relative to Th2 type, responses.

Figure 5 shows the cytokine profile in mice immunized with ovalbumin and adjuvanted with PPD and
35 PT(K9G129). PPD is an adjuvant that produces a Th1 immune response.

32

Splenocytes were obtained from mice immunized with
ovalbumin along with either S1(K9G129) rPT or PPD as
adjuvants. The spleen cells of four mice in each
treatment group were pooled and then restimulated in
5   vitro with ovalbumin alone (no adjuvant). The
supernatants were then harvested from these cultures
and the levels of IFN-γ and IL-4 were determined by
EIA. Similar IFN-γ:IL-4 ratios were obtained from
cultures derived from mice immunized with either .
10 S1(K9G129) or PPD as an adjuvant. As described above,
a higher ratio of IFN-γ:IL4 cytokines is characteristic
of a Th1 immune response.

Figure 6 shows the ovalbumin-specific IgG2a and
IgE responses of BALB/c mice immunized with ovalbumin
15 and either the S1(K9G129) PT analogue or PPD as
adjuvants. The bar graph indicates that immunization
with the S1(K9G129) PT analogue resulted in ratios of
ovalbumin-specific IgG2a:IgE ratios similar to those
obtained following immunization with PPD. As described
20 above, a high IgG2a:IgE ratio is characteristic of a
Th1 immune response. The results in Figures 5 and 6
thus indicate that adjuvanting with PT(K9G129) produces
a Th1 immune response in mice.

SUMMARY OF THE DISCLOSURE

25   In summary of this disclosure, the present
invention provides novel immunogenic compositions and
methods of immunization in which a genetically-
detoxified pertussis holotoxin, which may also be
immunogenic, is employed as a proteinaceous adjuvant in
30 place of conventional extrinsic adjuvants, particularly
alum, to achieve a modulated immune response to a non-
Bordetella antigen without the adverse side effects of
alum. Modifications are possible within the scope of
the invention.

TABLE 1a

Mutations introduced into Pertussis Toxin

Mutation Number	Mutation		
1.	ARG^9	->	▲9
2.	"	->	GLU^9
3.	"	->	LYS^9
4.	"	->	HIS^9
5.	ARG^{13}	->	▲13
6.	"	->	GLU^{13}
7.	ARG^9-ARG^{13}	->	▲9-13
8.	$ARG^9\ ARG^{13}$	->	$GLU^9\ GLU^{13}$
9.	ARG^{58}	->	GLU^{58}
10.	$ARG^{57}\ ARG^{58}$	->	▲57▲58
11.	TYR^{26}	->	ALA^{26}
12.	"	->	CYS^{26}
13.	CYS^{41}	->	ALA^{41}
14	"	->	SER^{41}
15.	CYS^{201}	->	ALA^{201}
16.	GLU^{129}	->	▲129
17.	::	->	GLY^{129}
18.	"	->	GLN^{129}
19.	"	->	ASP^{129}
20.	"	->	ASN^{129}
21.	"	->	LYS^{129}
22.	"	->	ARG^{129}
23.	"	->	HIS^{129}
24.	"	->	PRO^{129}
25.	"	->	CYS^{129}
26.	"	->	GLY^{129} II
27.	"	->	GLN^{129} II
28.	TYR^{130}	->	▲130
29.	"	->	PHE^{130}
30.	$GLU^{129}\ TYR^{130}$	->	$GLY^{129}\ ALA^{130}$
31.	$GLU^{129}\ TYR^{130}$	->	$GLN^{129}\ ALA^{130}$
32.	$GLU^{129}\ TYR^{130}$	->	$GLY^{129}\ PHE^{130}$
33.	$S3)LYS^{10}$	->	GLN^{10}
34.	$(S3)TYR^{92}\ LYS^{93}$	->	$ASN^{92}\ ARG^{93}$
35.	$(S3)LYS^{105}$	->	ASN^{05}
36.	$CYS^{41}\ CYS^{201}$	->	$ALA^{41}\ ALA^{201}$
37.	$CYS^{41}\ GLU^{129}$	->	$ALA^{41}\ GLY^{129}$
38.	$ARG^9\ GLU^{129}$	->	$GLU^9\ GLY^{129}$ II
39.	$ARG^9\ GLU^{129}$	->	$GLU^9\ GLN^{129}$ II
40.	$ARG^9\ GLU^{129}$	->	$GLU^9\ ARG^{129}$

TABLE 1a (con't)

Mutation Number	Mutation		
41.	ARG^9 GLU^{129} TYR^{130}	→	GLU^9 GLY^{129} ALA^{130}
42.	ARG^{13} GLU^{129}	→	GLU^{13} GLY^{129} II
43.	ARG^{13} GLU^{129}	→	GLU^{13} GLN^{129} II
44.	ARG^{13} GLU^{129} TYR^{130}	→	GLU^{13} GLY^{129} ALA^{130}
45.	ARG^9 GLU^{129}	→	Δ^9 GLN^{129} S-2730-1-1
46.	ARG^9 GLU^{129} TYR^{130}	→	Δ^9 GLY^{129} ALA^{130}
47.	ARG^{13} GLU^{129}	→	Δ^{13} GLN^{129}
48.	ARG^{13} GLU^{129} TYR^{130}	→	Δ^{13} GLY^{129} ALA^{130}
49.	GLU^{129}	→	GLY^{129}
	(S3)TYR^{92} LYS^{93}		(S3)ASN^{92} ARG^{93}
50.	Wild Type		
51.	Arg13	→	Lys13
52.	Arg58	→	His58
53.	Arg58	→	Lys58
54.	His35	→	Ala35
55.	Glu129	→	Ser129
56.	Tyr130	→	Ser130
57.	Arg58Glu129	→	GLu58Gly129
58.	Arg9Glu129	→	Lys9Gly129
59.	Arg9Arg58Glu129	→	Lys9Glu58Gly129
60.	(S3) Ile91Tyr92Lys93	→	Delete
61.	(S2) Thr91Arg92Asn93	→	Delete
62.	(S1) Glu129/		(S1) Gly129/
	(S3) Ile91Tyr92Lys93	→	S3(91-93) delete
63.	(S1) Arg58Glu129/		(S1) Glu58Gly129/
	S3(91-93)	→	S3(91-93) delete
64.	(S1) Arg9Glu129/		(S1) Lys9Gly129/
	S3(91-93)	→	S3(91-93) delete
65.	(S1) Arg9Arg58Glu129/		(S1)Lys9Glu58Gly129
	S3(91-93)	→	S3(91-93) delete
66.	(S2) Thr91Arg92Asn93/		S2(91-93) delete
	S3(91-93)	→	S3(91-93) delete
67.	(S3) TYR^{82}	→	ALA^{82}
68.	(S3) ΔILE^{91}	→	$\Delta^{91}\Delta^{92}\Delta^{93}$
69.	(S3) $TYR^{102}TYR^{103}$	→	$\Delta^{102}\Delta^{103}$
70.	(S3) ΔLYS^{105}	→	Δ^{105}
71.	(S3) LYS^{105}	→	ALA^{105}
72	(S3) LYS^{169}	→	ALA^{169}
73.	(S3) $TYR^{82}LYS^{169}$	→	$ALA^{82}ALA^{169}$
74.	(S4) TYR^4	→	ALA^4
75.	(S4) TYR^{21}	→	ALA^{21}
76.	(S4) $LYS^{54}LYS^{57}$	→	$ALA^{54}ALA^{57}$

TABLE 1a (con't)

Mutation Number	Mutation		
77.	(S3) TYR^{82}(S4)LYS4^{54}LYS^{57}	->	(S3) ALA^{52}/(S4)ALA^{54}ALA^{57}
78.	(S1) GLU^{129}/(S3)TYR^{82}	->	(S1) GLY^{129}/S3(▲82)
79.	(S1) GLU^{129}/S3(ILE^{91}TYR^{92}LYS^{93})	->	(S1) GLY^{129}/(S3)▲^{91}▲^{92}▲^{93}

Notes:

Amino acid numbering corresponds to positions in the native subunits.

All mutations are in subunit S1 unless specified as being in S2, S3 or S4.

II denotes use of an alternative codon.

▲ denotes deleted residue(s).

Wild type refers to PT expressed from the unmutated TOX operon in B. parapertussis.

TABLE 1b

In vitro characterization of pertussis toxin analogues obtained from recombinant *B. parapertussis*.

Mutation Number	Residual Toxicity(%)	ADPR Activity(%)	S1 Epitope
1.	0.2	ND	−
2.	0.1	0.2	+/−
3.	0.1	ND	+++
4.	0.2	0.1	+++
5.	0.3	ND	−
6.	5.0	ND	+++
7.	0.4	0.1	−
8.	0.1	0.9	−
9.	0.7	0.6	+++
10.	0.4	ND	−
11.	0.5	ND	+
12.	6.0	ND	ND
13.	0.3	0.4	−
14.	1.4	ND	ND
15.	0.2	0.1	−
16.	0.1	ND	++
17.	0.1	0.3	++++
18.	0.02	0.1	+/−
19.	0.7	2.5	++
20.	0.1	0.3	++
21.	0.3	0.2	−
22.	0.1	ND	−
23.	0.2	ND	+
24.	0.2	ND	−
25.	0.4	ND	
26.	0.1	0.3	+++
27.	0.02	0.1	+/−
28.	0.2	0.1	−
29.	12.0	ND	+++
30.	0.2	0.6	−
31.	0.4	ND	−
32.	1.0	ND	+++
33.	100	ND	+++++
34.	50	100	+++
35.	20	ND	+++

TABLE 1b (con't)

Mutation Number	Residual Toxicity(%)	ADPR Activity(%)	S1 Epitope
35.	20	ND	++++
36.	0.2	0.1	-
37.	0.1	0.1	-
38.	0.1	0.1	-
39.	0.1	ND	-
40.	0.1	ND	-
41.	0.2	ND	-
42.	0.5	ND	-
43.	3.0	ND	-
44.	0.3	ND	-
45.	0.4	ND	-
46.	0.2	0.1	-
47.	0.5	ND	-
48.	0.4	0.3	-
49.	0.2	0.1	++++
50.	100	100	++++
51.	14.0		++++
52.	35.0		++++
53.	13.0		++++
54.	0.2		++
55.	0.6		++++
56.	29.0		++++
57.	0.1		++
58.	<0.001	<0.001	+++
59.	0.1		+
60.	12.0		++++
61.	100.0		++++
62.	0.03	0.2	+++
63.	0.1		+
64.	0.1		+++
65.	0.1		+
66.	10.0		+
67.	7.2	96	ND
68.	4.6	108	ND
69.	9.6	98	ND
70.	8.1	57	ND
71.	94	71	ND
72.	102	71	ND
73.	5.2	92	ND
74.	46	125	ND
75.	84	91	ND
76.	9.6	55	ND
77.	1.5	97	ND
78.	0.04	0.10	ND
79.	0.04	0.16	ND

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Notes:

Residual toxicity is the ratio of the apparent PT concentration determined by the CHO cell clustering assay to the actual concentration of PT mutant determined by ELISA expressed as a percentage.

ADPR activity is the extent of ADP-ribosylation of bovine transducin catalysed by a PT analogue, relative to that catalysed by an equal concentration of wild-type PT, expressed as a percentage.

S1 epitope refers to the expression of an immunodominant S1 epitope recognized by a specific monoclonal antibody PS21 (ATCC HB 10299 deposited November 30, 1989), as compared with the wild-type PT (+++++).

ND denotes not determined.

TABLE 2

Functional amino acid residues in pertussis toxin for mutation

Subunit	Residues	Preferred Replacement
S1	Phe-23	Asp or Glu
	Ser-48	Ala
	Val-51	Ile
	Gln-127	Ala or Asp
	Leu-131	Lys or Arg
	Gly-199	Val or Gln
	Ala-200	Ile
	Phe-235	Glu
S2	His-15	Ala or Thr
	Gln-16	Ala or Thr
	Trp-52	Val
	Glu-66	Ala or Lys
	Asp-81	Ala or Ser
	Leu-82	Ala or Glu
	Lys-83	Glu
	Ser-104	Ala
	Arg-125	Ala
	Ser-147	Thr
	Arg-150	Ser
	Lys-151	Ser
S3	Gln-15	Ala or Thr
	Gln-16	Ala or Thr
	Tyr-82	Ala or Val
	Arg-83	Glu
	Ser-104	Ala
	Arg-125	Ala
	Arg-150	Ser
	Arg-151	Ser
S4	Asp-1	Ala
	Tyr-4	Ala or Val
	Gly-60	Val
	Ser-61	Ala
	Glu-65	Ala
	Arg-69	Ala
	Thr-88	Val
	Pro-93	Ala
	Asp-54	Glu
	Thr-51	Tyr
	Thr-55	Tyr
	Gly-58	Val
S5	Ser-62	Ala

TABLE 3

Immunogenicity of the Pertussis toxin component of a DTP acellular vaccine and an acellular DTP vaccine containing a genetically detoxified PT analog

Vaccine Formulation	Anti-pertussis toxin IgG Reactive titre	Reciprocal CHO cell neutralizatoin titre
DTP acellular vaccine	1,363,678	50.8
Recombinant acellular vaccine	1,363,678	40.3

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CLAIMS

What we claim is:

1. An immunogenic composition, which comprises:
    a genetically-detoxified pertussis holotoxin, and
    at least one other, non-Bordetella, antigen, wherein said genetically-detoxified pertussis holotoxin is present in an amount sufficient to modulate an immune response to said other antigen in the absence of an extrinsic adjuvant.

2. The composition of claim 1 wherein said immune response is selected from a humoral response, a cellular response and both a humoral and a cellular response.

3. The composition of claim 1 wherein said modulated immune response to said other antigen is selected from an enhanced IgG response, a cellular response and both an enhanced IgG and a cellular response.

4. The composition of claim 1 wherein said other antigen provides a protective immune response to at least one pathogen.

5. The composition of claim 4 wherein said pathogen is selected from the group consisting of bacterial, viral and parasitic pathogens.

6. The composition of claim 5 wherein said at least one pathogen is selected from the group consisting of Corynebacterium diphtheriae, Clostridium tetani, paramyxoviridae, haemophilus, influenza, hepatitis, meningococci, streptococci, schistosoma and trypanosoma.

7. The composition of claim 6 wherein said genetically-detoxified pertussis holotoxin is immunoprotective.

8. The composition of claim 4 wherein said other antigen is a cancer-associated antigen.

9. The composition of claim 8 wherein said cancer is selected from melanoma, bladder, lung, cervical and prostate cancer.

10. The composition of claim 1 wherein such other antigen comprises inactivated tumor cells or membrane fraction thereof.

11. The composition of claim 10 wherein said cells are inactivated by irradiation.

12. The composition of claim 1 wherein at least one amino acid is removed or replaced in said genetically-detoxified pertussis holotoxin.

13. The composition of claim 12 wherein multiple amino acids are removed and replaced in said genetically-detoxified pertussis holotoxin.

14. The composition of claim 12 or 13 wherein said at least one amino acid is selected from the group consisting of (S1) ARG^9, ARG^{13}, TRP^{26}, ARG^{58} and GLU^{129}.

15. The composition of claim 13 wherein said multiple amino acids are (S1) ARG^9, GLU^{129}.

16. The composition of claim 15 wherein said multiple amino acids are replaced (S1) ARG^9 to LYS^9 and GLU^{129} to GLY^{129}.

17. The composition of claim 1 wherein said genetically-detoxified pertussis holotoxin is selected from those listed in Tables 1a, 2 and 3.

18. The composition of claim 1 containing at least one additional Bordetella antigen.

19. The composition of claim 18 wherein said Bordetella antigen is selected from the group consisting of agglutinogens, FHA and pertactin.

20. The immunogenic composition of claim 1 which is formulated in the substantial absence of an extrinsic adjuvant as a vaccine for human or animal administration.

21. The immunogenic composition of claim 20 wherein said composition exhibits a decreased IgE response.

22. The immunogenic composition of claim 1 which is formulated in the substantial absence of alum as a multivalent vaccine comprising said genetically-detoxified pertussis holotoxin in an immunoprotective

form and amount and diphtheria toxoid and tetanus toxoid
as said at least one other, non-Bordetella, antigen.

23. The immunogenic composition of claim 22 which
further comprises at least an additional Bordetella
antigen.

24. The immunogenic composition of claim 23 wherein said
additional Bordetella antigen is selected from the group
consisting of agglutinogens, FHA and pertactin.

25. A method of obtaining a modulated immune response to
an antigen in a host, which comprises:

    administering at least one non-Bordetella antigen to
said host, and

    coadministering to said host a genetically-
detoxified holotoxin in an amount sufficient to modulate
an immune response to said other antigen in the absence
of an extrinsic adjuvant.

26. The method of claim 25 wherein said immune response
is selected from a humoral response, a cellular response
and both a humoral and a cellular response.

27. The method of claim 25 wherein said modulated immune
response to said non-Bordetella antigen is selected from
an enhanced IgG response, a cellular response and both an
enhanced IgG and a cellular response.

28. The method of claim 25 wherein said administration
and coadministration are effected by administering a
composition as claimed in claim 1 to 22 to said host in
the absence of an extrinsic adjuvant.

29. The method of claim 25 wherein said host is a human.

Novel Pertussis Vaccine Formulation
Potentiation of Serum IgE Levels

FIG.1.

Novel Pertussis Vaccine Formulation
Antigen Specific IgG Responses

FIG.2.

FIG.3.

Novel Pertussis Vaccine Formulation
Combination Vaccines

IgG Reactive Titre

Vaccine Formulation

DTP whole cell   DTP acellular   rDTP acellular

☐ Anti-tetanus txd   ☐ Anti-Diphtheria txd

FIG.4.

Murine Cytokine Profiles
Potentiation of Th 1 Phenotypes

FIG.5.

Antigen-Specific Immunoglobulin
Subclass Profiles

FIG.6.
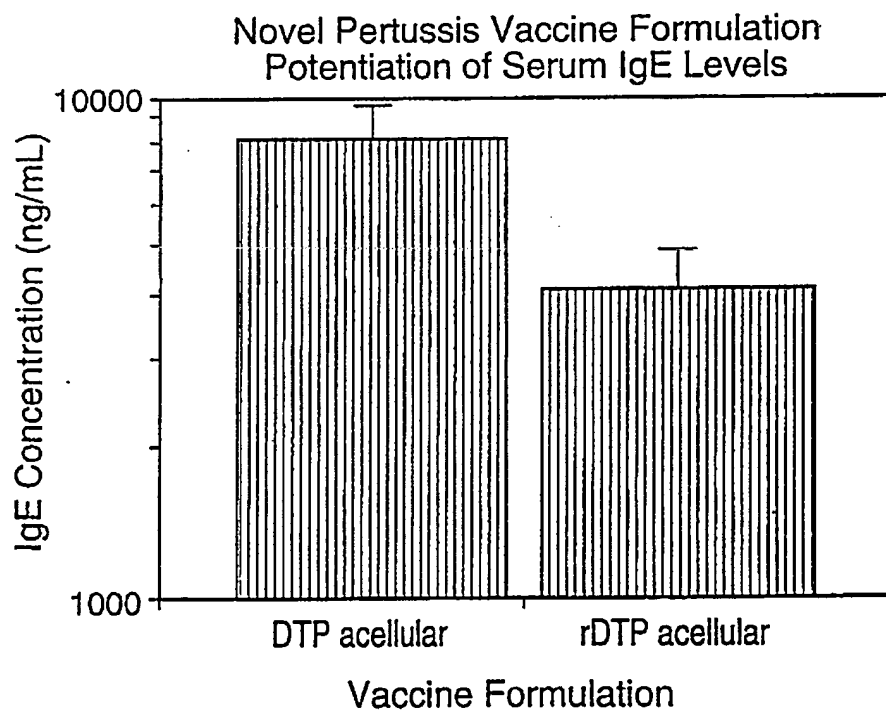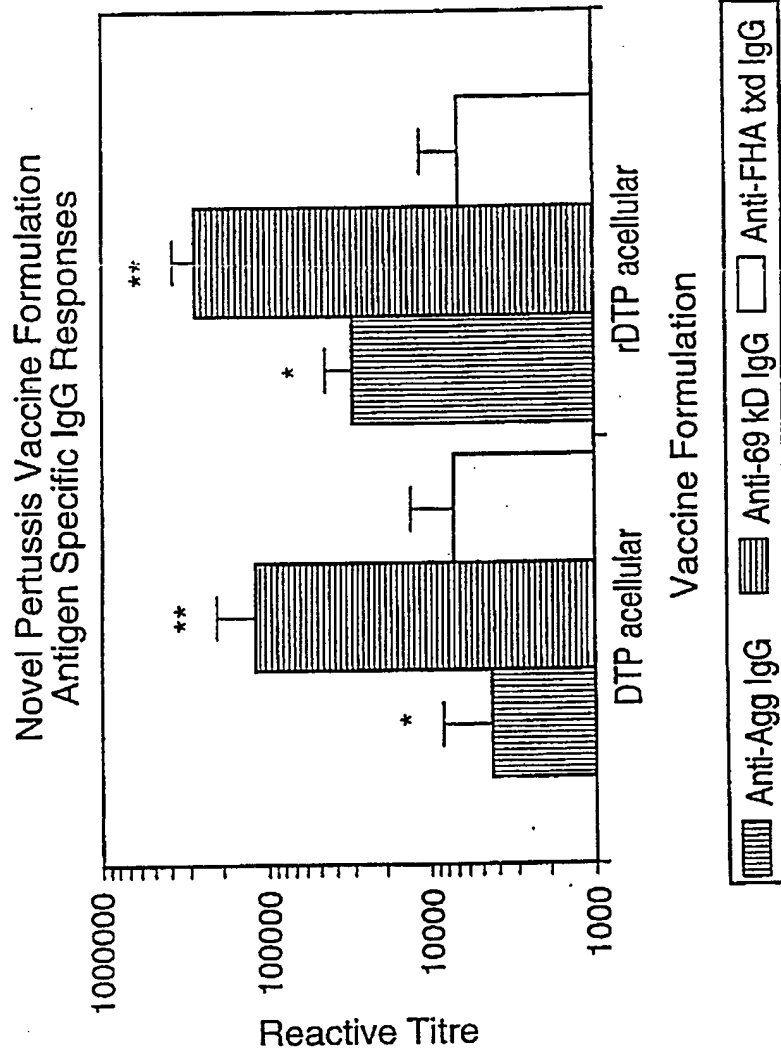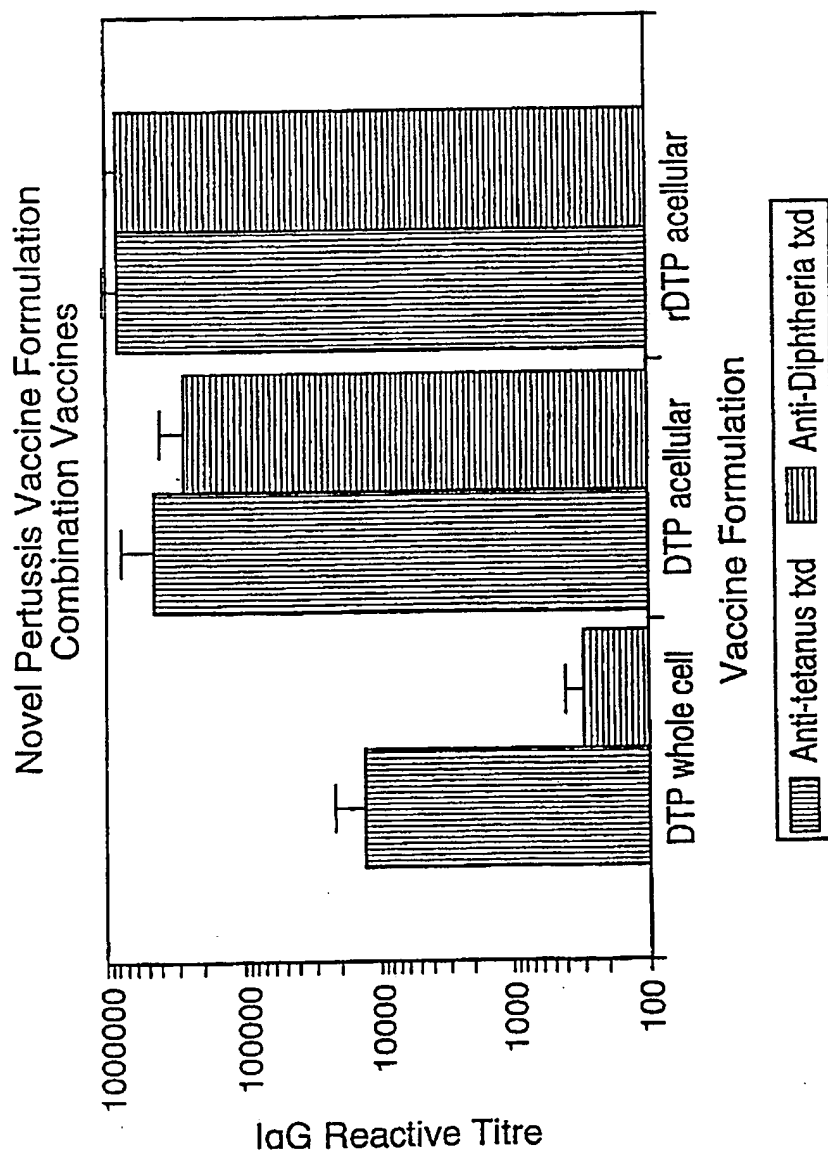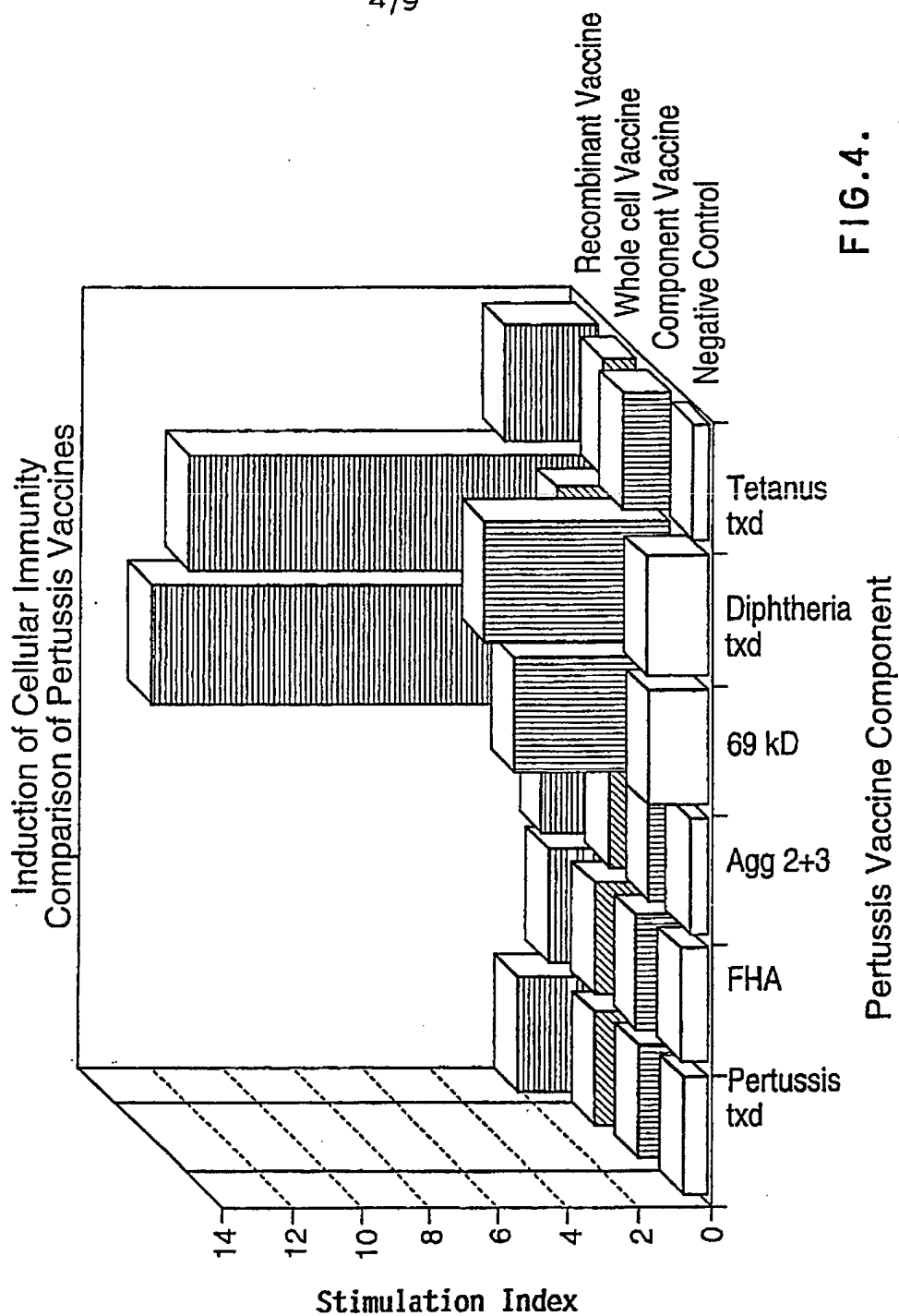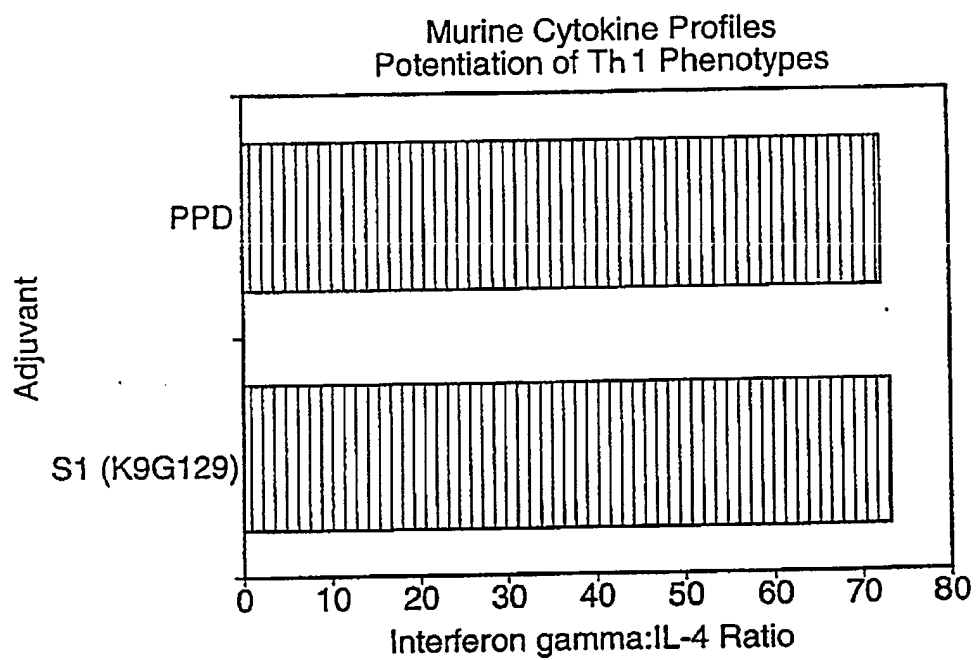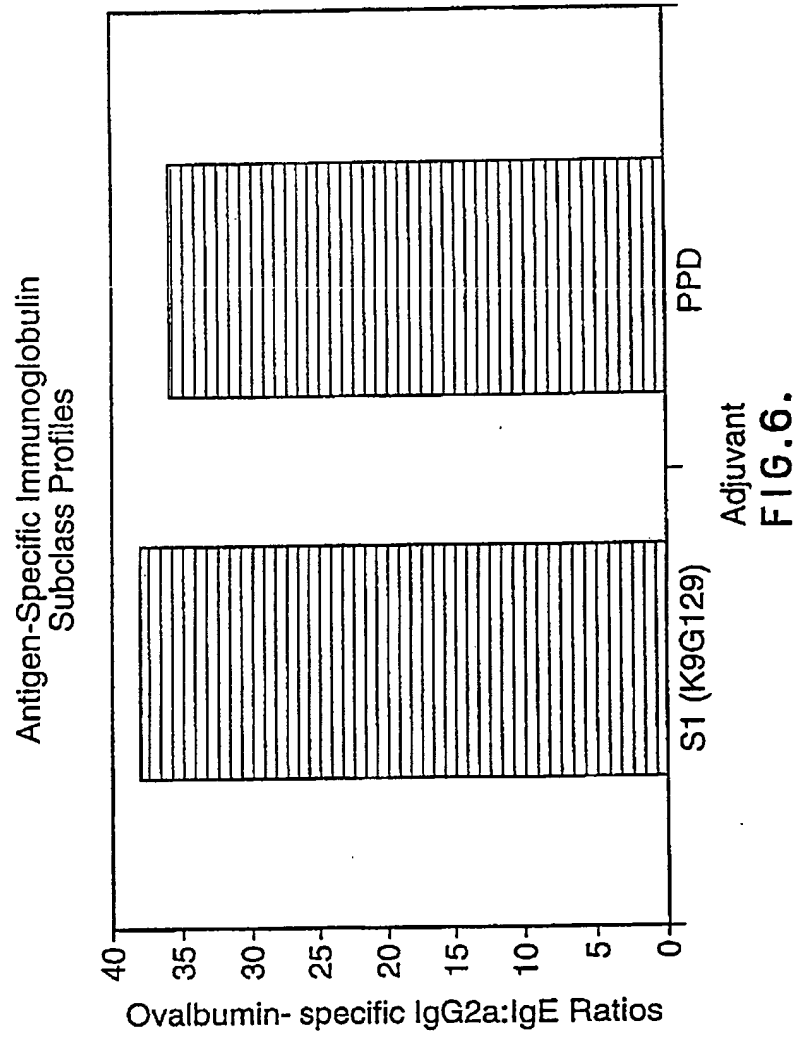
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Number of tumor-free mice at 30 days (N=5)
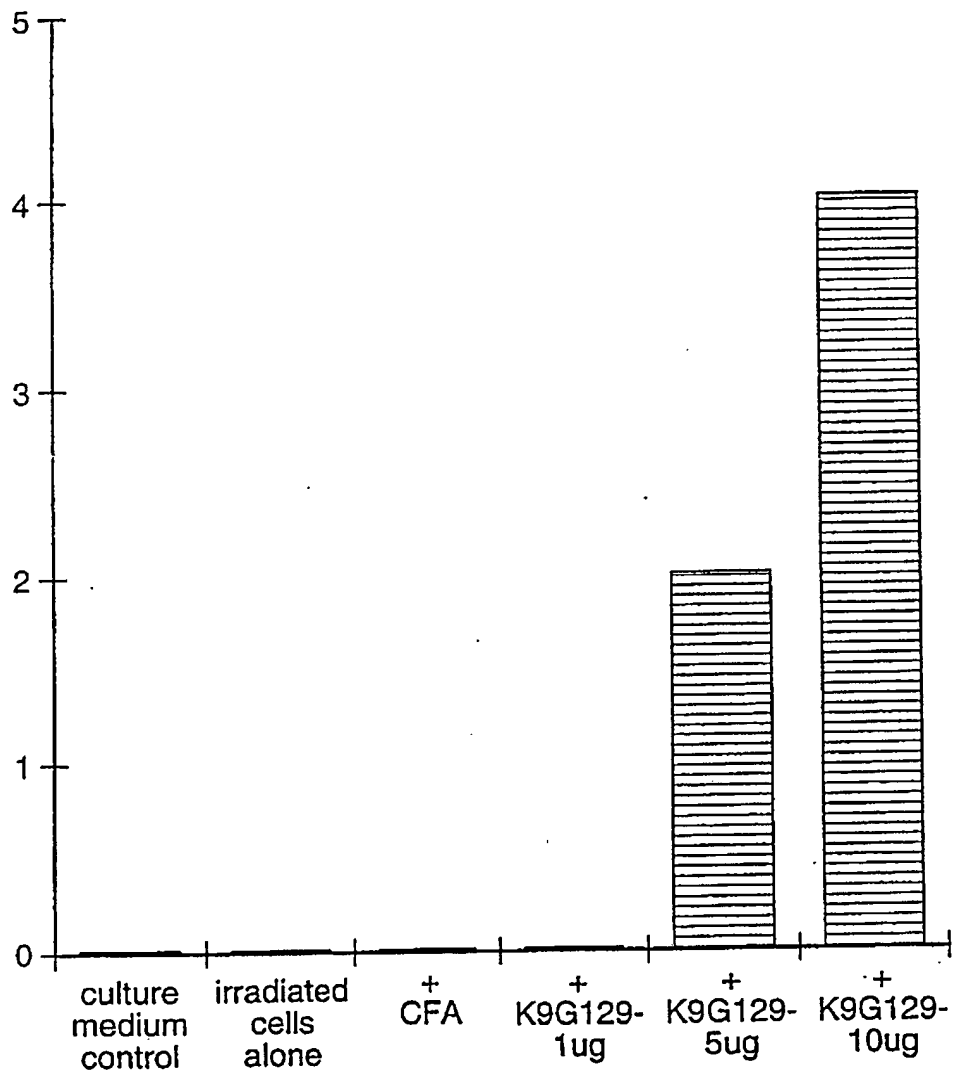
FIG.7.

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Adjuvant Effect of K9G129

FIG.8.

Tumor volume at day 22

FIG.9.